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Directed Chromosomal Integration and Expression of the Reporter Gene *gusA3* in *Lactobacillus acidophilus* NCFM[▽]

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Lactobacillus acidophilus **NCFM is a probiotic microbe that survives passage through the human gastrointestinal tract and interacts with the host epithelium and mucosal immune cells. The potential for *L. acidophilus* to express antigens at mucosal surfaces has been investigated with various antigens and plasmid expression vectors. Plasmid instability and antibiotic selection complicate the possibility of testing these constructs in human clinical trials. Integrating antigen encoding genes into the chromosome for expression is expected to eliminate selection requirements and provide genetic stability. In this work, a reporter gene encoding a β-glucuronidase (GusA3) was integrated into four intergenic chromosomal locations. The integrants were tested for genetic stability and GusA3 activity. Two locations were selected for insertion downstream of constitutively highly expressed genes, one downstream of *slpA* (LBA0169), encoding a highly expressed surface-layer protein, and one downstream of phosphopyruvate hydratase (LBA0889), a highly expressed gene with homologs in other lactic acid bacteria. An inducible location was selected downstream of *lacZ* (LBA1462), encoding a β-galactosidase. A fourth location was selected in a low-expression region. The expression of *gusA3* was evaluated from each location by measuring GusA3 activity on 4-methyl-umbelliferyl-β-D-glucuronide (MUG). GusA3 activity from both highly expressed loci was more than three logs higher than the *gusA3*-negative parent, *L. acidophilus* NCK1909. GusA3 activity from the *lacZ* locus was one log higher in cells grown in lactose than in glucose. The differences in expression levels between integration locations highlights the importance of rational targeting with gene cassettes intended for chromosomal expression.**

Lactic acid bacteria (LAB) have demonstrated significant potential as vaccine delivery vehicles, mostly by using plasmids, expressing bioactive compounds at mucosal surfaces where they may stimulate an appropriate immune response (4, 27, 36). LAB offer several advantages over current systemic vaccination routes. Several strains may act as natural adjuvants, possibly eliminating the use of toxic adjuvants common with systemic vaccines. Strains have also been identified that offer protection against degradation during passage through the gastrointestinal tract to deliver vaccines to the mucosal surface, where they may induce both mucosal and systemic immunity (36).

The ability of *Lactobacillus acidophilus* NCFM to survive passage through the gastrointestinal tract and interact with intestinal and immune cells indicates the potential of this probiotic strain to be used as a vaccine delivery vehicle (19, 31). *L. acidophilus* NCFM has previously been used to express a protective antigen from a plasmid against anthrax, conferring protection to mice in a lethal anthrax challenge (24). Bioactive compounds must be expressed at a level high enough to elicit a desired immune response (36). While the multiple copy number of plasmids may seem advantageous for expression, plasmid instability and the selective pressure required for maintenance complicates their use in human clinical applications (25, 28). Integrating genes into the chromosome for expression is expected to eliminate selection requirements and provide genetic stability. While the levels of expression from plasmids have been higher than those from single-copy chromosomal integration cassettes (6, 23), few chromosomal locations have been investigated for integration, and alternative sites may provide stable, high levels of expression.

Several systems have previously been devised to integrate genes into the chromosomes of LAB. Phage machinery has been used to integrate genes of interest into the *attB* site present in some lactic acid-producing species. Selective markers were flanked by *six* sites, enabling their removal with β-recombinase (22). Several systems have demonstrated integration by two recombination events, the first integrating the entire plasmid, and the second removing the plasmid but leaving the gene of interest in place. One demonstration of this was with *Lactobacillus casei* CECT 5276 (Lac^-), a strain containing a frameshift mutation in *lacF* that enabled efficient selection of a second recombination event at this site that rendered the strain Lac^+ (12). Double recombination was also used to replace the *thyA* gene with interleukin 10 (IL-10) in the chromosome of *Lactococcus lactis*, creating a strain that could be used in human clinical trials to treat inflammatory bowel disease (33). Similar gene replacement strategies have been used to integrate a gene in both *Lactobacillus gasseri* and *Lactobacillus plantarum* (6, 14). Additionally, double recombination methods have been used with *L. acidophilus* NCFM to inactivate genes with an internal deletion (26). However, the use of double recombination is limited in efficiency by the extensive screening required to identify a double recombination event or the need for a screenable mutation at the site of integration. Additionally, the current demonstrations of integration often disrupt native genes, which may be detrimental to the strain (6, 14).

Recently, counterselectable systems have been devised that enable efficient selection of a second recombination event (10,

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32). The previously described *upp*-counterselectable gene replacement system is compatible with *L. acidophilus* NCFM, a fully sequenced probiotic bacteria with available global gene expression profiles (1, 3, 10). The work presented here demonstrates integration of a *gusA3* reporter gene, encoding a β-glucuronidase, into four intergenic locations throughout the NCFM chromosome using the *upp*-counterselectable gene replacement system, resulting in a range of potential expression levels (7). This work elucidates the possibilities for integrating genes into multiple chromosomal locations with minimal to no deletion of native DNA, enabling selection of one or more integration sites that can provide maximum expression. Comparison of *gusA3* expression from chromosomal locations with *gusA3* expressed from a plasmid indicates that rational integration location targeting with gene cassettes has the potential to produce higher expression levels.

MATERIALS AND METHODS

Bacterial strains and propagation conditions. All strains used in this work are listed in Table 1. *Lactobacillus acidophilus* NCFM-derived strains were propagated in de Man, Rogosa, and Sharpe (MRS; Becton, Dickinson and Company [BD], Sparks, MD) broth at 37°C under static conditions, or on MRS agar (1.5% [wt/vol]; Fisher Scientific, Pittsburg, PA) under anaerobic conditions. *Escherichia coli*-derived strains were propagated in brain heart infusion (BHI; BD) broth at 37°C with aeration or on BHI agar aerobically. Erythromycin (Em), kanamycin (Kn), and chloramphenicol (Cm), all from Fisher, and penicillin G (Sigma-Aldrich, St. Louis, MO) were added to growth media as necessary.

DNA cloning techniques. Chromosomal DNA was extracted with the ZR bacterial fungal DNA miniprep kit (Zymo Research Corporation, Orange, CA), and plasmids were isolated from *E. coli* with the Qiagen QIAprep spin miniprep kit (Qiagen Inc., Valencia, CA). Primers were designed manually or with Clone Manager Professional 9 (Sci-Ed Software, Cary, NC) and synthesized by Integrated DNA Technologies (IDT; Coralville, IA). Restriction enzymes were from Roche Molecular Biochemicals (Indianapolis, IN). The 1-kb Plus DNA ladder was from Invitrogen (Carlsbad, CA). Following electrophoresis, DNA was isolated from agarose gels with the Zymo gel DNA recovery kit and purified, if necessary, with the Zymo Clean & Concentrator-5 (Zymo Research Corporation). High Fidelity DNA polymerase (Roche Molecular Biochemicals) was used for cloning, and Choice-*Taq* Blue DNA polymerase (Denville Scientific Inc., Metuchen, NJ) was used for screening. T4 DNA ligase (New England BioLabs, Ipswich, MA) or the Fast-Link DNA ligation kit (Epicentre Biotechnologies, Madison, WI) were used for ligations per the manufacturer's instructions.

NCK1831 *E. coli*-competent cell production and subsequent transformation was performed as described by Hanahan, with modifications to the RF1 buffer (10 mM potassium acetate, 50 mM magnesium chloride, 100 mM rubidium chloride, 10 mM calcium chloride, 15% [wt/vol] glycerol, pH 8.0) and RF2 buffer (10 mM morpholineethanesulfonic acid [MOPS], 75 mM calcium chloride, 10 mM rubidium chloride, 15% [wt/vol] glycerol, pH 6.5) (13). Following ligation, cells were recovered in BHI broth for 1 to 2 h at 37°C with aeration and then plated on BHI agar containing 150 μg/ml Em and 40 μg/ml Kn and grown for 24 to 48 h at 37°C.

Preparation of competent *Lactobacillus* cells and subsequent transformation was performed as previously described, with modifications (21, 34, 35). Briefly, a 100-ml culture of NCK1910 cells was grown in MRS medium at 37°C containing 5 μg/ml Cm for 3 h, and then 100 μg/ml of penicillin G was added. The culture continued to grow for another 2 h. The cells were pelleted at 4°C at 1,717 × *g* and resuspended in 40 ml ice-cold 3.5× SMEB buffer (1 M sucrose and 3.5 mM magnesium chloride, pH 7). The cells were pelleted and washed in 3.5× SMEB buffer two more times and then resuspended in 1 ml 3.5× SMEB buffer, resulting in competent cells. Approximately 500 ng plasmid DNA (derivatives of pTRK935) was added to a 200-μl aliquot of competent cells on ice and transformed by electroporation in a cold 0.2-cm cuvette at 2.5 kV, 25 μFD, and 400 Ω. Cells were recovered in MRS overnight and then plated on MRS medium containing 2 μg/ml of both Em and Cm.

Selection of chromosomal locations for *gusA3* integration. Chromosomal locations for integration of *gusA3* were selected using a heat map depicting the expression of each gene in *L. acidophilus* NCFM in response to growth in eight carbohydrate sources (3). Intergenic sites were chosen to minimize interference with the native genetic code. TransTermHP was used to find Rho-independent transcription terminators (17). Basic Local Alignment Search Tool (BLAST) was used when determining the homology of integration locations in other lactic acid bacteria (15).

TABLE 1. Bacterial strains and plasmids used in this study

Strain and plasmid	Genotype/characteristics	Reference
E. coli strains		
NCK1831	EC101 RepA$^+$ host strain for pORI-based plasmids; Kn^r	20
NCK1911	EC101 harboring pTRK935	10
NCK1978	MC1061 harboring pTRK892	8
L. acidophilus strains		
NCK56	*L. acidophilus* NCFM	
NCK1829	*L. acidophilus* NCFM harboring pTRK892	8
NCK1909	*L. acidophilus* NCFM Δ*upp* strain; W$^+$	10
NCK1910	NCK1909 harboring pTRK669, host for gene targeting insertion or replacement	10
NCK2136	NCK1909 with *gusA3* chromosomal insertion downstream of LBA0169 (*slpA*)	This study
NCK2139	NCK1909 with *gusA3* chromosomal insertion downstream of LBA1462 (*lacZ*)	This study
NCK2171	NCK1909 with *gusA3* chromosomal insertion downstream of LBA0889 (*eno*)	This study
NCK2190	NCK1909 with *gusA3* chromosomal insertion downstream of LBA0645 (low expression)	This study
Plasmids		
pTRK669	Temperature-sensitive helper plasmid; RepA$^+$; Cm^r	29
pTRK892	Source of *gusA3*; Em^r	8
pTRK935	Counterselective integration vector with *upp* expression cassette; *lacZ′*; Em^r	10
pTRK1020	pTRK935 containing *gusA3* between the upstream and downstream sequence flanking the LBA0169 integration site	This study
pTRK1022	pTRK935 containing *gusA3* between the upstream and downstream sequence flanking the LBA1462 integration site	This study
pTRK1039	pTRK935 containing *gusA3* between the upstream and downstream sequence flanking the LBA0889 integration site	This study
pTRK1045	pTRK935 containing *gusA3* between the upstream and downstream sequence flanking the LBA0645 integration site	This study

Integration of *gusA3* into the chromosome with the *upp*-counterselective gene replacement system. The *upp*-based counterselective gene replacement system, previously used to create gene deletions, was used with modifications to integrate *gusA3* into four locations in the *L. acidophilus* NCFM chromosome (10). A plasmid was constructed for each integration, starting with the integration plasmid, pTRK935. For integration downstream of LBA0169, the upstream and downstream sequences flanking the chromosomal integration site in NCFM were obtained with PCR using the 169up and 169dn forward and reverse primers (Table 2) and triple ligated directionally into the multiple cloning site of pTRK935. This was repeated for the locations downstream of LBA1462, LBA0889, and LBA0645, using the primer sets corresponding to each gene

TABLE 2. Cloning and sequencing primers for *L. acidophilus* NCFM chromosomal *gusA3* insertions

Primer (ORF)	Primer sequence[a] (5′–3′)		Size of product (bp)
	Forward	Reverse	
Cloning primers			
169up	CAAGCGATCGACCAAGGTCAAGTTAATG	CAAGGAATTCTAGCGTTAGTGCTAC	692
169dn	CAAGGAATTCAGGCAGAGCGAAAGCTCTG	CAAGGTCGACGACCGACGGTACTATTTATC	603
1462up	CAAGGTCGACTCTCGTCTGTATATTCTAAC	CAAGGCGGCCGCCGAACGAAAATGTCCGGCCT	623
1462dn	CAAGGCGGCCGCACCTTATTTATTTGATCT ACGG	CAAGCGATCGTCTATGAACGCAATATTCC	752
889up	CAAGCGATCGATAGCGATTGTATTAGTAAC	CAAGGCGGCCGCACCGAGTACAATTTCAAC	616
889dn	CAAGGCGGCCGCATTAATCGTGGTCAACATC	CAAGGTCGACTTTACACGAATCAATCATC	626
645up	CAAGCGATCGGATGTTGCTCAGGTAGATAG	CAAGGCGGCCGCTCACTAAAGAGGTCGAGAGTATG	624
645dn	CAAGGCGGCCGCCTATATGGTATTTTTATGT GCACTGGCA	CAAGGTCGACGAGTAAACATCGAGAAATTG	613
p892_Gus1	CAAGGAATTCTAAGAAGGCTGAATTCTAC	CAAGGAATTCTGAGCACGATTATTTG	1,864
p892_Gus2	CAAGGCGGCCGCTAAGAAGGCTGAATTCTAC	CAAGGCGGCCGCTGAGCACGATTATTTG	1,864
Screening primers			
p935mcs	TGAAATACCGCACAGATG	ACACAGGAAACAGCTATG	
169	GTTGCTTCACTTACTAATG	CTTATCCCTTTAGGATATTCAG	
1462	GAATCCATGAGTCGAAATATC	TTTAGGGTCAAAGACTAAGG	
889	GTTGTTGTTCCTGTAGGTAAGATTG	CCACACTTTGAAGAAGGTTCTTG	
645	AGGCTAAATATTAATGATTTACC	GAAGAGAACGAAATTTAAC	

[a] Enzyme restriction sites are underlined.

number (Table 2). The ligation mixtures were transformed into NCK1831, and successful ligations were selected by blue-white screening on plates that were each spread with 0.8 mg X-Gal (5-bromo-4-chloro-3-inodyl-β-D-glucuronic acid) and 4 mg IPTG (isopropyl-β-D-thiogalactopyranoside).

Each plasmid was then digested between the flanking sequences, with EcoRI or NotI as appropriate, and treated with SuperSAP-phosphatase (NEB) for ligation with the *gusA3* gene. The *gusA3* gene was obtained from pTRK892 by PCR using either p892_Gus primer set 1 (EcoRI restriction site) or 2 (NotI restriction site) (Table 2). The *gusA3* product from both primer sets contained 37 bp upstream of the start codon, including a ribosomal binding site, and 30 bp following the stop codon. Successful ligations were screened with colony PCR using the p935mcs primer set with the p892_Gus primer sets (Table 2) to obtain clones with *gusA3* in the correct directional orientation. Plasmids with positive clones were extracted and confirmed with sequencing prior to transformation into NCK1910.

The NCK1910 transformants were screened using colony PCR analysis using the p935mcs primer set (Table 2). Successful transformants were integrated into the NCFM chromosome as previously described by Goh et al. (10). Briefly, cultures were transferred (1% inoculum) three times and grown to stationary phase each time (~24-h incubations) in a 42°C water bath to select for loss of the pTRK669 helper plasmid. Loss of pTRK669 and single-crossover integrants of the targeting plasmid rendered the transformants Em resistant, 5-flouro-uracil (5-FU) sensitive, and Cm sensitive. Em-resistant and Cm-sensitive colonies were transferred one to three times in MRS medium and grown to stationary phase each time (~24-h incubations). Clones that had lost the plasmid through a second homologous crossover event were rendered 5-FU resistant and were selected by plating on semidefined medium with glucose (GSDM) (16) and 100 μg/ml 5-FU (10). Colonies were screened for *gusA3* integration with primers upstream and downstream to the crossover event, using the primers corresponding to the number of the upstream gene in each location. Integrants were confirmed by sequencing these amplicons (Table 2).

Measurement of GusA3 activity. GusA3 activity was measured by the fluorescence produced upon hydrolysis of the substrate 4-methyl-umbelliferyl-β-D-glucuronide (MUG). Assays were performed as described previously (8, 30), with modifications. Cells were grown (10 ml) to log phase (optical density at 600 nm [OD_{600}] of 0.6 to 0.8) and collected by centrifugation at 1,717 × *g* for 10 min at room temperature. Cells were resuspended in semisynthetic medium (SSM) (2) with 1% lactose (wt/vol) or 1% glucose (wt/vol) for 1 h at 37°C, then collected by centrifugation at 4°C, washed twice in 10 ml ice-cold GUS buffer (100 mM sodium phosphate buffer with 2.5 mM EDTA, pH 6.0), and resuspended in 0.5 ml of ice-cold GUS buffer. Cells were then homogenized with glass beads in three 1-min cycles with a Mini-BeadBeater-8 (BioSpec Products, Inc., Bartlesville, OK), with 1 min on ice between cycles. Cell extracts (CFEs) were collected by centrifugation at 8,600 × *g* for 2 min and held on ice.

The Bradford (Sigma) (5) assay was performed per the manufacturer's instructions using a 96-well microtiter plate reader to determine protein concentrations of CFEs. Bovine serum albumin (BSA) in GUS buffer was used to produce a standard curve (0 to 1 mg protein/ml).

Samples were serially diluted from 0.1 to 0.00001 mg/ml protein, and 100 μl of each dilution was incubated at 37°C for 1 min. The samples were vortexed with 100 μl 2 mM MUG and incubated at 37°C for 5 min. The reaction was stopped by vortexing with 800 μl 0.2 M Na_2CO_3 stop buffer. Fluorescence was measured using a 96-well microtiter plate reader (excitation 355 nm, emission 460 nm). 4-Methylumbelliferone (4-MU) in GUS/stop buffer (1:4 ratio) was used to produce a standard curve (0 to 250 nM 4-MU). GusA3 activity was reported as nanomoles of 4-MU liberated per minute per mg protein.

Stability of *gusA3* integrants. The four strains containing *gusA3* chromosomal integrations were analyzed for stability after transferring the cultures five times (~24-h incubations) in MRS medium at 37°C (1% inoculum; ~35 generations). The DNA was extracted, and PCR was used to confirm the presence of a *gusA3* integrant or a wild-type band in each strain using primers upstream and downstream of the integration event (Table 2). GusA3 activity in glucose was also measured to confirm stability.

The NCK 1829 strain carrying the pTRK892 plasmid was also transferred five times (~24-h incubations) in MRS medium without Em selection and plated on both MRS agar and MRS agar with 2 μg/ml Em. Stability of pTRK892 was calculated as the percentage of CFU resistant to Em.

RESULTS

Selection of integration sites. The global gene expression profile of *L. acidophilus* NCFM enabled selection of sites for integration and expression of the reporter gene *gusA3* (3). Integration sites were selected based on several criteria. First, *gusA3* was not integrated with a promoter, and polycistronic expression of *gusA3* from the promoter of the upstream gene was necessary for expression. However, gusA3 was integrated with a ribosomal binding site for translation. Second, integration sites were selected downstream of genes with different expression levels (high

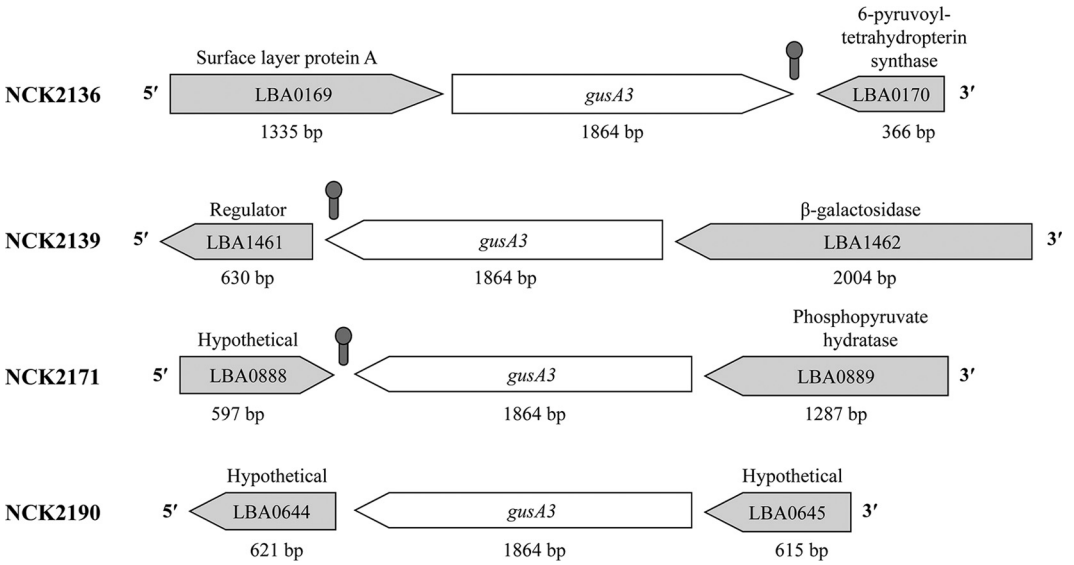

FIG. 1. Intergenic locations selected for *gusA3* integration into the *L. acidophilus* NCFM chromosome. The symbols downstream of *gusA3* in NCK2136, NCK2139, and NCK2171 indicate a terminator. The figure is drawn approximately to scale.

and low, constitutive and inducible) in order to investigate using gene expression profiles to select integration locations. Finally, in order to limit gene disruption and regulatory effects on other genetic elements, insertion locations were intergenic, preferably between a stop codon and a terminator.

Two locations were selected downstream of genes with high constitutive expression. The first location was downstream of *slpA* (LBA0169), encoding a surface-layer protein (Fig. 1, NCK2136). The second location was downstream of a phosphopyruvate hydratase, or enolase (*eno*) (LBA0889), which encodes a protein sharing >93% identity with a phosphopyruvate hydratase in several other lactic acid bacteria, as well as some similarity in the arrangement of surrounding genes (Fig. 1, NCK2171, and Fig. 2). TransTermHP predicted a transcriptional terminator (100% confidence) downstream of both genes (17). The *gusA3* gene was inserted after the stop codon and before the terminator of both *slpA* (strain NCK2136) and *eno* (strain NCK2171) with minimal intergenic sequence deletion.

A third location was selected downstream of *lacZ* (LBA1462), encoding a β-galactosidase inducible by lactose

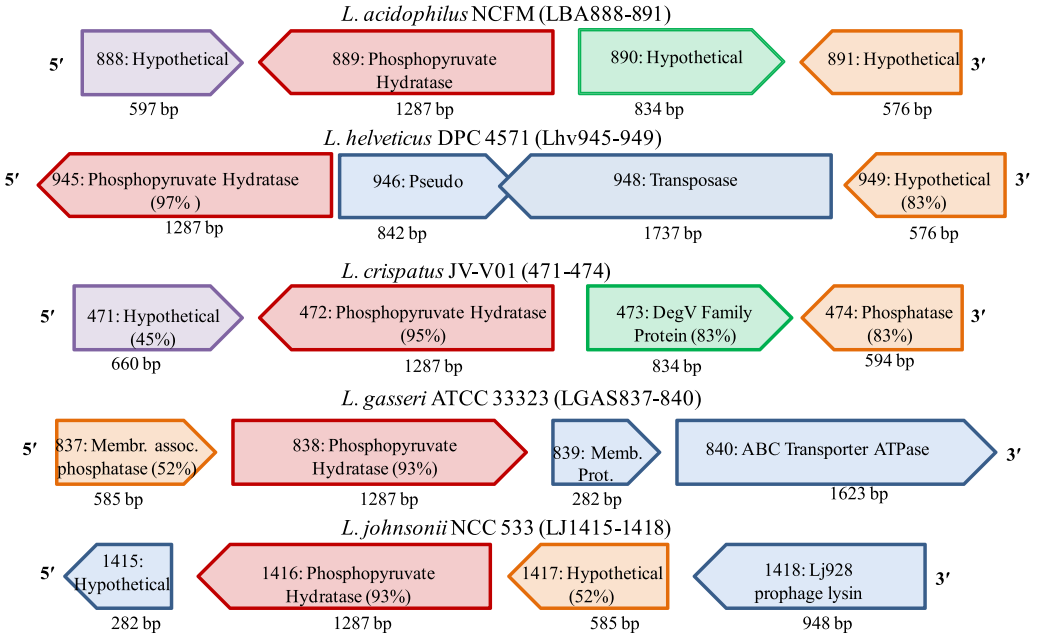

FIG. 2. Similarity of genomic arrangement surrounding phosphopyruvate hydratase between *L. acidophilus* NCFM and several closely related lactic acid bacteria. Genes shown in the same color share percent protein identity, indicated in parentheses, with the gene of the same color in *L. acidophilus*. Genes in blue do not show percent protein identity to the NCFM genes displayed here. The figure is drawn approximately to scale.

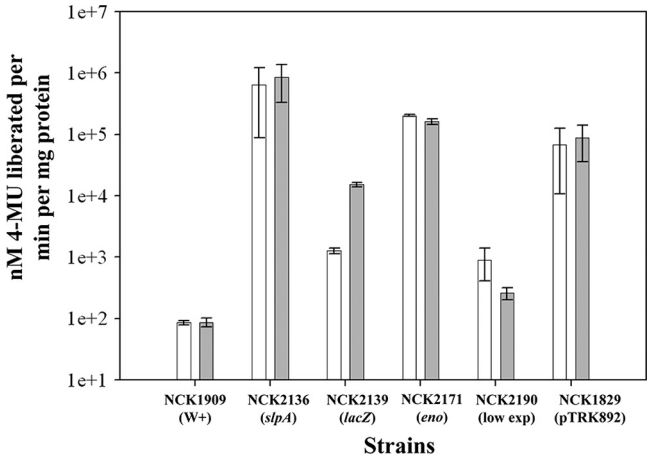

FIG. 3. Activity of GusA3 from chromosomal locations compared to plasmid expression in cells grown in glucose (white bars) or lactose (gray bars). The results shown here are the averages of three independent assays ± one standard deviation.

(Fig. 1, NCK2139). TransTerm HP predicted a transcriptional terminator (74% confidence) downstream of *lacZ*. This terminator was disrupted in order to construct the integration plasmid. However, the resulting strain (NCK2139 [*lacZ*]) grew at the same rate as NCK2136 (*slpA*), NCK2171 (*eno*), and NCK1909 W^+ (data not shown).

Selection of the fourth location (low expression) was more difficult due to high A/T intergenic regions in many potential integration sites. The *gusA3* gene was integrated downstream of LBA0645, a hypothetical protein with 75% identity to adenylate cyclase in *Lactobacillus kefiranofaciens* zw3 (strain NCK2190) (Fig. 1, NCK2190). The location downstream of LBA0645 is the only one of the four integration locations that did not encode a predicted terminator. Additionally, the high A/T content in the intergenic region between LBA0645 and LBA0644 (another hypothetical protein with low expression) was not favorable to design primers for construction of the integration plasmid. Primer design resulted in the deletion of 60 of the 66 intergenic base pairs. A growth curve determined that NCK2190 grew at the same rate as NCK1909 W^+ and the other integration mutants (data not shown); however, the pellet appeared to be less compact after growth.

GusA3 activity. The GusA3 activity of CFEs from the four *gusA3* integration strains (NCK2136 [*slpA*], NCK2139 [*lacZ*], NCK2171 [*eno*], and NCK2190 [low expression]) was assessed after 1 h of exposure to either glucose or lactose. Both NCK2136 (*slpA*) and NCK2171 (*eno*) produced high constitutive GusA3 activity in both carbohydrate sources. GusA3 activity was four logs higher in NCK2136 (*slpA*) and three logs higher in NCK2171 (*eno*) than in the *gusA3*-negative parent (Fig. 3).

The GusA3 activity of NCK2139 (*lacZ*) was induced by one log in lactose compared to glucose (Fig. 3). The basal level of GusA3 activity measured from NCK2139 (*lacZ*) in glucose was consistent with the activity measured from NCK2190 (low expression), which was expected to produce, at most, a low level of GusA3 activity.

The GusA3 activity from the chromosomal integration strains was compared with activity from the pTRK892 plasmid (strain NCK1829), where *gusA3* was under the control of a phosphoglycerate mutase (*pgm*) promoter, previously shown to have high, constitutive expression (3, 8). The GusA3 activity of NCK2136 (*slpA*) was nearly 10-fold higher than the activity of NCK1829(pTRK892) in both glucose and lactose (Fig. 3). GusA3 activity was similar between NCK2171 (*eno*) and NCK1829.

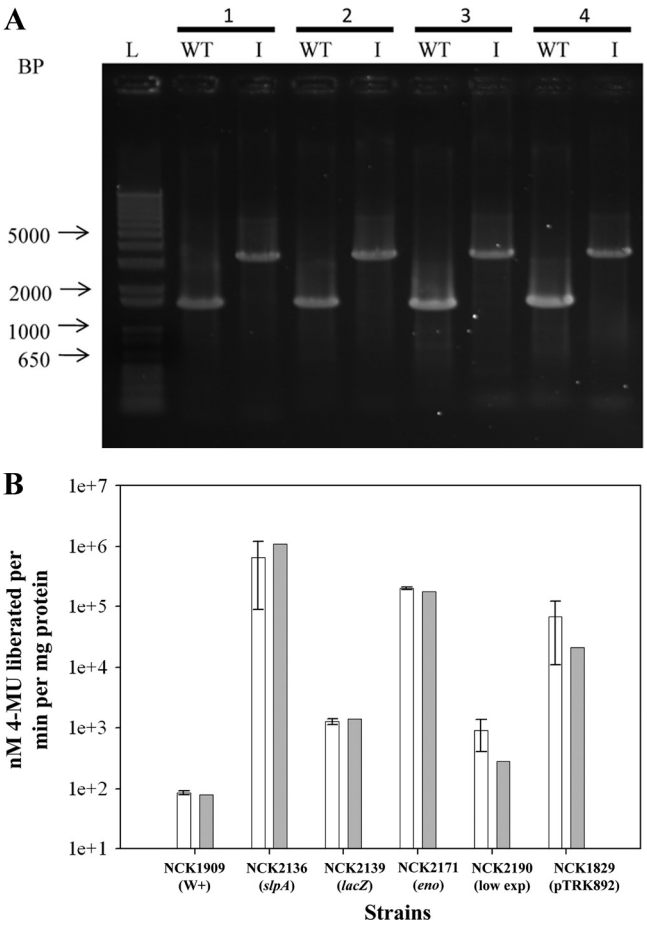

FIG. 4. Stability of *gusA3* in each chromosomal location after 35 generations. (A) PCR analysis confirming each integration was stable after 35 generations. L, ladder; BP, base pairs; WT, wild type (NCK1909); I, integrant. Lane 1, NCK2136; 169 primers; WT amplicons, 1,566 bp; I amplicons, 3,430 bp. Lane 2, NCK2139; 1,462 primers; WT amplicons, 1,583 bp; I amplicons, 3,463 bp. Lane 3, NCK2171; 889 primers; WT amplicons,1,531 bp; I amplicons, 3,403 bp. Lane 4, NCK2190; 645 primers; WT amplicons, 1,574 bp; I amplicons, 3,395 bp. (B) GusA3 activity in glucose after seven generations (white bars) and 35 generations (gray bars). The results shown for seven generations are the averages of three independent assays ± one standard deviation. One GusA3 assay was completed after 35 generations for each integrant construct, and PCR assays were conducted to confirm the absence of wild-type amplicons. For the plasmid construct NCK1829(pTRK892), genetic stability was evaluated by plate count comparisons of Em^r versus Em^s colonies.

Stability of integrations. PCR was used to determine the stability of the *gusA3* integrants after 35 generations. The absence of a wild-type band in each integration strain indicated that the gene was stable in all four chromosomal locations (Fig. 4A). Additionally, the GusA3 activity, measured to confirm

stability results, was similar in NCK2136 (*slpA*), NCK2139 (*lacZ*), and NCK2171 (*eno*) to the activity measured after only seven generations (Fig. 4B). Although no wild-type band appeared in the NCK2190 (low expression) strain, the GusA3 activity was lower after 35 generations.

The pTRK892 plasmid was not stably maintained in NCK1829 when the strain was cultivated for 35 generations in antibiotic-free media. Only 38.5% of the cells maintained erythromycin resistance, indicating a 61.5% plasmid loss. The GusA3 activity of NCK1829 decreased similarly (Fig. 4B).

DISCUSSION

In clinical settings, the amount of gene expression obtained is often critical to achieving the desired response. The availability of the genome sequence of *L. acidophilus* NCFM and global gene expression data enabled selection of chromosomal locations with high, constitutive expression levels for integration of the reporter gene, *gusA3* (1, 3). Additionally, the development of the *upp*-counterselection system for *L. acidophilus* NCFM enabled *gusA3* integration into multiple chromosomal locations, followed by excision of the plasmid backbone and associated selection markers, with minimal screening (10). This work demonstrated that the ability to select integration sites likely to produce high expression enabled achievement of integration cassettes with expression levels similar to or higher than that obtained from a plasmid.

Plasmids have been used to express foreign genes in lactobacilli for decades. Multicopy expression plasmids promote higher expression levels than can normally be achieved from single-copy chromosomal loci. However, previous groups have measured both higher (6, 23) and lower (14) expression from a plasmid than from a chromosomal integrant. It is possible that integrating a promoter along with a gene, instead of relying only on a native promoter, may further enhance expression levels. Hols et al. attributed the higher expression observed from a chromosomal integrant to simultaneous expression from both the upstream promoter and the promoter integrated with the gene (14).

The pTRK892 plasmid used in this work is a low-copy-number plasmid that expressed *gusA3* from the *pgm* promoter (8). This promoter was previously shown through global transcriptional expression analysis of *L. acidophilus* to have high constitutive activity, similar to the promoters for *slpA* and *eno* (3). The higher GusA3 activity obtained from the *slpA* promoter than from the pTRK892 plasmid demonstrates the high expression achievable with integration into rationally selected locations.

Plasmid pTRK892 is a derivative of pGK12 (8, 18), which replicates by the rolling circle mechanism (RCR) (9, 25). These plasmids contain single-stranded intermediates that are unstable and may generate internal deletions (9). The addition of foreign DNA to such RCR plasmids can lead to stability issues due to their larger size. Additionally, the requirement for erythromycin to maintain plasmid selection complicates the use of these constructs in clinical settings. Without erythromycin selection, pTRK892 was not as stable as the chromosomal *gusA3* integrations. While other plasmids may be more stable, the work presented here suggests that not only does single-copy chromosomal integration have the potential for high expression, it has the potential for greater stability than that obtained from plasmids.

Both *slpA* and *eno* are highly expressed genes with upstream and downstream genes on the complement strand that have much lower expression. Additionally, there is a terminator downstream of both of these genes. The ability to integrate *gusA3* downstream of highly expressed genes is not universal. An attempt had been made to integrate *gusA3* between two highly constitutively expressed ribosomal proteins (LBA0294 and LBA0295) to obtain high expression of *gusA3*. This insertion site did not result in a stable *gusA3* integrant, and high expression over this region seemed to be unstable for the integration cassette. This suggests that the low expression regions upstream and downstream of both *slpA* and *eno*, along with the downstream terminators, may have facilitated stable integration and expression of *gusA3* in these locations and that similar genomic positions may be optimal for integrant targeting.

The surface layer protein encoded by *slpA* has previously demonstrated immunogenic properties, with the ability to bind to a dendritic cell-specific receptor, DC-SIGN, and stimulate anti-inflammatory T-cell responses (19). The ability of SlpA to interact with immune cells and stimulate T-cell responses suggests that the *slpA* promoter expresses at a level capable of producing immunogenic levels of protein. The high level of activity of GusA3 at this location substantiates its potential for stable integration and expression of antigenic proteins.

Phosphopyruvate hydratase, or enolase, is an enzyme in the glycolysis pathway that catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate. While the *eno* promoter did not produce GusA3 activity at the level of the *slpA* promoter, it is comparable to activity from pTRK892. The pTRK892 plasmid backbone was previously used to deliver a dendritic cell targeting anthrax protective antigen to mice (24). The amount of protein delivered from pTRK892 was able to elicit protection against a lethal anthrax challenge, indicating that this amount of expression is adequate for clinical purposes. Additionally, the delivery of multiple biotherapeutic proteins from the same strain would require multiple integration sites to be available. The high percent protein identity shared between this phosphopyruvate hydratase and similar proteins in other LAB suggests that this site has potential as an integration target in other strains.

Lactose operons have been used previously for inducible expression, to control both expression of genes from plasmids and chromosomal locations (8, 12). The use of an inducible operon is beneficial in situations where control of gene expression is required or in specific environments, such as the use of the inducible lactose operon in dairy applications. Although the terminator was disrupted to construct NCK2139 (*lacZ*), no sequence downstream of the terminator was altered. The absence of anomalies during growth of NCK2139 suggests that maintaining the integrity of the sequence downstream of the terminator may have prevented further regulatory effects on other genetic elements.

The LBA0645 hypothetical protein is upstream of a second hypothetical protein (LBA0644). In order to design constructs for integration of *gusA3* into this intergenic region, 60 of the 66 bp were deleted, and it is possible that translation of LBA0644 was disrupted. The resulting strain, NCK2190 (low expression), grew at a rate similar to that of the other integration strains and NCK1909 W^+. *L. acidophilus* NCFM is a self-

aggregating strain that results in a pellet at the bottom of a test tube after growth (11). The NCK2190 pellet was present but less compact after growth in comparison to that of the other strains. LBA0644 has 74% identity to a dithiol-disulfide isomerase in *Lactobacillus crispatus* ST1 and does not encode domains that indicate a role in aggregation. This region was chosen to obtain only basal expression levels of GusA3 activity to demonstrate (i) the range of expression that may be obtained throughout the genome and (ii) the possible consequences of gene integration without careful location selection. Low-level expression was achieved by NCK2190, as expected. This location will not likely be used to express bioactive compounds in the future unless low expression is desirable. The interference with cellular aggregation observed in this strain further confirms that locations between stop codons and terminators are more ideal sites for gene integration.

This work demonstrates the integration of the reporter gene, *gusA3*, into four locations throughout the *L. acidophilus* NCFM chromosome using the *upp*-counterselectable gene replacement system. Integration locations were selected based on global gene expression profiles. The range of expression achieved correlated with the gene expression data, indicating that this is an acceptable method for selecting integration locations. Additionally, this work constructed chromosomal integration cassettes that achieve both higher expression than that obtained from a plasmid as well as a low, basal level of expression, establishing the importance of rational targeting when selecting integration locations.

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